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TITLE: Targeted, On-Demand Charge Conversional Nanotherapeutics for Advanced Prostate Cancer

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14. ABSTRACT

In this project, we have proposed to design and test an innovative nanotherapeutic system to attack prostate cancer occurred in skeletal tissue. The proposed targeted, bio-responsive nanotherapeutics are based on peptide-functionalized diblock copolymers of poly(ethylene glycol) and poly(trimethenecarbonate) (PEG-PTMC) as the drug carrier, by using docetaxel as the therapeutic agent. During the past funding period, copolymers with predetermined terminal functionality, molecular weights and chemical compositions have been synthesized, and the physicochemical properties of nanotherapeutics including drug loading capacity and drug release profile have been investigated. The peptide functionalized nanotherapeutics possess high HA binding affinity, indicating their good bone-targeting capacity. We have investigated and evaluated biological activities of the drug-loaded nanotherapeutics in terms of cellular uptake and tumor inhibition in cultured prostate cancer cells. We have also initiated bone uptake and retention of the proposed nanotherapeutics in mice. For the future study, we will further perform therapeutic efficacy studies in vivo, such as survival study and bone-related therapeutic evaluation of bone-targeted nanotherapeutics to improve therapy for advanced prostate cancers.

15. SUBJECT TERMS
Prostate cancer, bone metastasis, bone-targeting, drug delivery system

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1. **INTRODUCTION:**

Advanced prostate cancer frequently leads to skeletal complications that are very difficult to treat and result in pain, bone fractures, nerve compression, morbidity, and often mortality, and it is considered to be an incurable disease. Current therapeutic options are usually palliative in nature and there is a clear need for better treatment options for bone metastatic prostate cancers. This proposal addresses such an important public health need. Specifically, this project proposes a novel osteotropic, bio-responsive, and prostate-specific drug delivery system for advanced prostate cancer induced bone metastases. The proposed nanotherapeutic system using a multilevel targeting strategy could lead to a more effective approach to attack prostate cancers. The nanotherapeutics possess favorable pharmacological features to improve bioavailability. Additionally, such a therapeutic strategy to deliver therapeutic agents into metastatic bone microenvireoments may lead to a potential synergistic effect and improve overall therapeutic efficacy.

2. **KEYWORDS:** Prostate cancer, bone metastasis, bone-targeting, drug delivery system

3. **ACCOMPLISHMENTS:**

What were the major goals of the project?

In this application, we intend to design and test an innovative nanotherapeutic system to attack prostate cancer occurred in skeletal tissue. The proposed targeted, bio-responsive nanotherapeutics are based on a peptide functionalized diblock copolymers of poly(ethylene glycol) and poly(trimethenecarbonate) (PEG-PTMC) as the drug carrier, by using docetaxel as the therapeutic agent. Copolymers with predetermined terminal functionality, molecular weights and chemical compositions will be synthesized to fine-tune the amphiphilicity, solubility and drug loading capacity of the carriers. We will evaluate the physicochemical properties of nanotherapeutics including size, drug loading capacity, and drug release profile. We will also perform studies to determine *in vivo* HA binding affinity, *in vivo* bone uptake and retention of the nanotherpeutics. The nanotherapeutics will be extensively investigated in cultured prostate cells to determine biological properties in terms of CTSK-triggered cellular uptake and anti-tumor efficacy. Finally, the proposed nanotherapeutics will be tested in mice with prostate cancer induced bone metastases.

To accomplish the objectives of the proposed research, the following tasks will be achieved:

Task 1: Synthesize and characterize the nanotherapeutic constructs.

Task 2: Determine *in vitro* HA binding affinity, *in vivo* bone uptake and retention of the Asp8-containing nanoparticles.

Task 3: Investigate and evaluate biological activities of the docetaxel-loaded nanotherapeutics in terms of cellular uptake and tumor inhibition in cultured prostate cancer cells.

Task 4: Therapeutic evaluation of the proposed nanotherapeutics and appropriate controls in mice with prostate cancer induced bone metastases.

What was accomplished under these goals?

We have completed the synthesis and characterization of block copolymer based drug carriers for bone-targeted, bio-responsive nanotherapeutics for advanced prostate cancer therapy, and the drug-loaded nanoparticles (i.e. nanotherpeutics) were achieved by conjugation of functional moiety (CK(DUPA)GHPGGPQAsp8) with a bone tropism domain, a CTSK-cleavable substrate, and a prostate-specific ligand. During the past reporting period, we have been focusing on the studies of CTSK responsiveness of nanotherapeutics *in vitro*. A variety of experiments have been performed to investigate and evaluate biological activities of the nanotherapeutics in terms of cellular uptake and tumor inhibition in cultured prostate cancer cells by using dynamic light scattering (DLS), confocal laser scanning microscopy (CLSM), fluorescence-activated cell sorting (FACS), and MTT cell proliferation assay. In addition, we have confirmed that Asp8-containing nanoparticles are favorable for bone uptake and retention in comparison with Asp8-free nanoparticles in vivo.

In this project, we have hypothesized that Asp8 could be cleaved from NPs in the presence of cathepsin K, a biomarker in metastatic skeletal tissue, thus unmasking negatively charged nanotherapeutics and making DUPA ligand available to attack prostate cancer cells. In order to test our hypothesis, nanoparticles were prepared via dialysis method by using Pep-b-PEG-b-PTMC or mPEG-PTMC polymers. Cathepsin K was pre-incubated at 37°C for 5 min to activate the enzyme in the active site, followed by addition of the polymeric micelles for CTSK digestion. Surface charge and size of NPs before/after enzymatic incubation were monitored by dynamic light scattering. As shown in Figure 1, following treatment with CTSK, the zeta potential of Pep-b-PEG-b-PTMC NPs increased dramatically from -21.2 ± 0.9 mV to -2.9 ± 0.1 mV over 24-h (Figure 1 A). In contrast, the zeta potential of mPEG-b-PTMC NPs remained stable from -1.6 ± 0.7 mV to -1.8 ± 0.3 mV. There was no significant changes of the particle size during the period of enzymatic incubation of NPs (Figure 1 B), suggesting that enzymatic digestion did not alter the integrity of NPs after Asp8 out shell removal by CTSK.

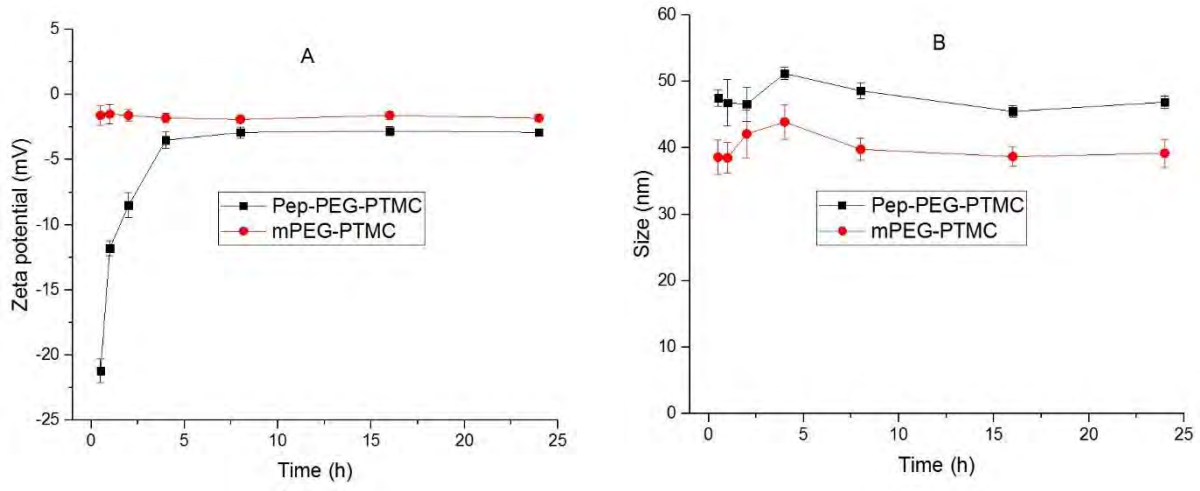


Figure 1: DLS monitoring of NPs for zeta potential (A) and particle size (B) of polymeric micelles upon co-incubation with 150 nM CTSK.

Confocal laser scanning microscopy (CLMS) was used to investigate the cellular uptake of polymeric micelles with or without CTSK treatment. The nanoparticles assembled from Pep-b-PEG-b-PTMC were loaded with Cy5.5 fluorescence indicator, followed by incubating with C4-2 prostate cancer cells in a cleavage buffer in the presence or absence of cathepsin K. Following 3-h incubation, polymeric micelles were evaluated by CLMS observation. There was little evident cellular uptake of fluorescence-labelled micelles from Pep-b-PEG-b-PTMC without CTSK treatment (Data not shown). The poor cellular uptake of untreated micelles was expected, because it was difficult for the highly negatively charged NPs to be internalized by cells. However, once these polymeric micelles were pretreated with CTSK, significant fluorescence enhancement was observed in the CLSM images (Figure 2). This was likely due to interaction between PMSA receptors in the cell membranes and DUPA ligands in NPs, thus facilitating the cellular uptake of NPs. These results indicated that nanoparticles assembled from Pep-b-PEG-b-PTMC were hardly taken up by cells. However, once the nanoparticles localize in CTSK-rich conditions, CTSK-triggered Asp8 detachment from outer shells of NPs could result in DUPA exposure to cell membrane, therefore facilitating cell entry of nanoparticles via receptor-mediated endocytosis.

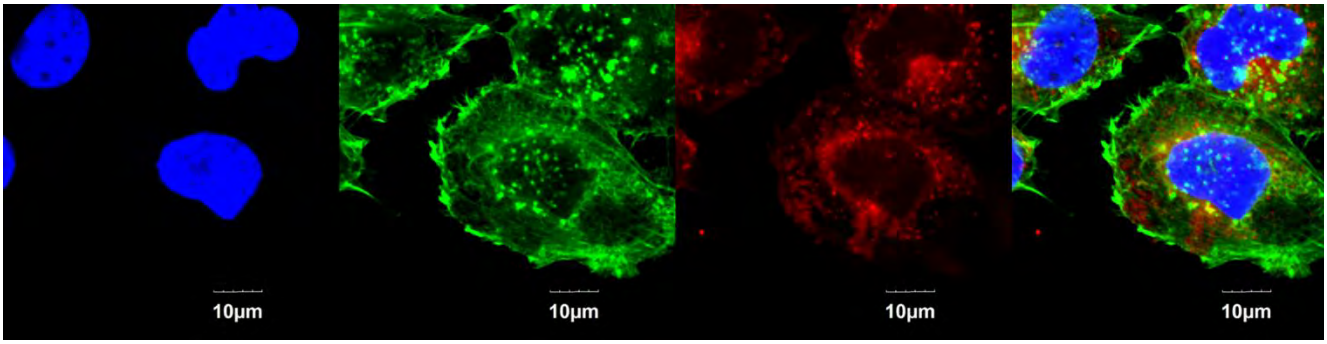


Figure 2. Cellular uptake of Cy5.5-loaded nanoparticles in C4-2 prostate cancer cells. Nanoparticles were treated with 150 nM CTSK followed by incubation with cells at 37 ^{o}C for 3-h. Cy5.5 was shown as red fluorescence. DAPI (4',6-diamidino-2-phenylindole, blue) and Alexa Fluor®488 phalloidin (green) were used to stain cell nuclei and F-actin, respectively. Cells were imaged using a 60 × water-immersion objective.

The observation in CLSM was further confirmed by FACS analysis. Compared with the cellular uptake of nanotherapeutics without CTSK treatment, remarkably enhanced intracellular fluorescence was detected after the nanotherapeutics were pre-incubated with CTSK (Figure 3).

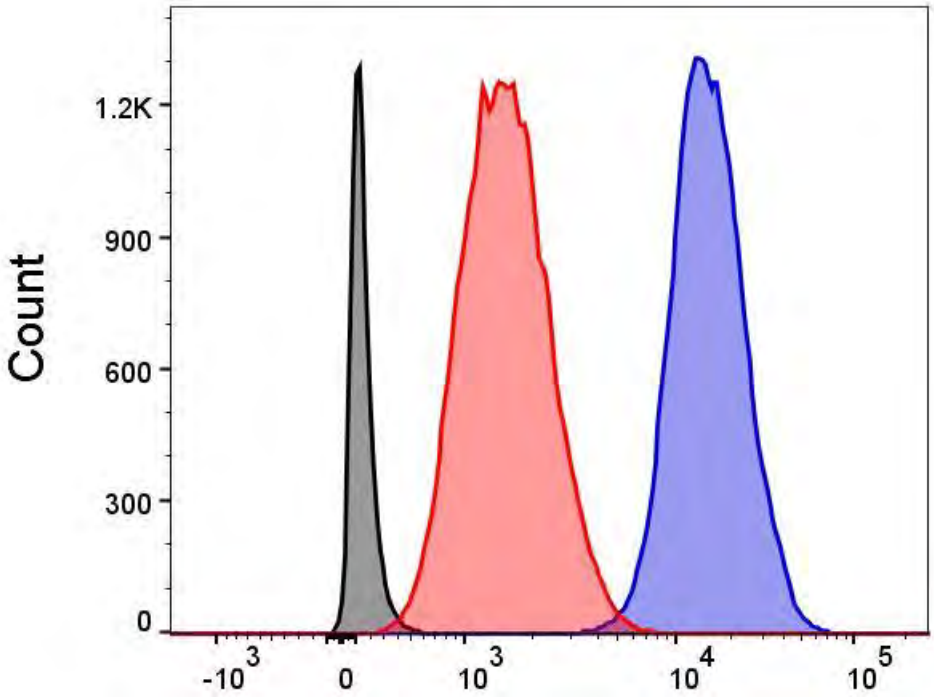
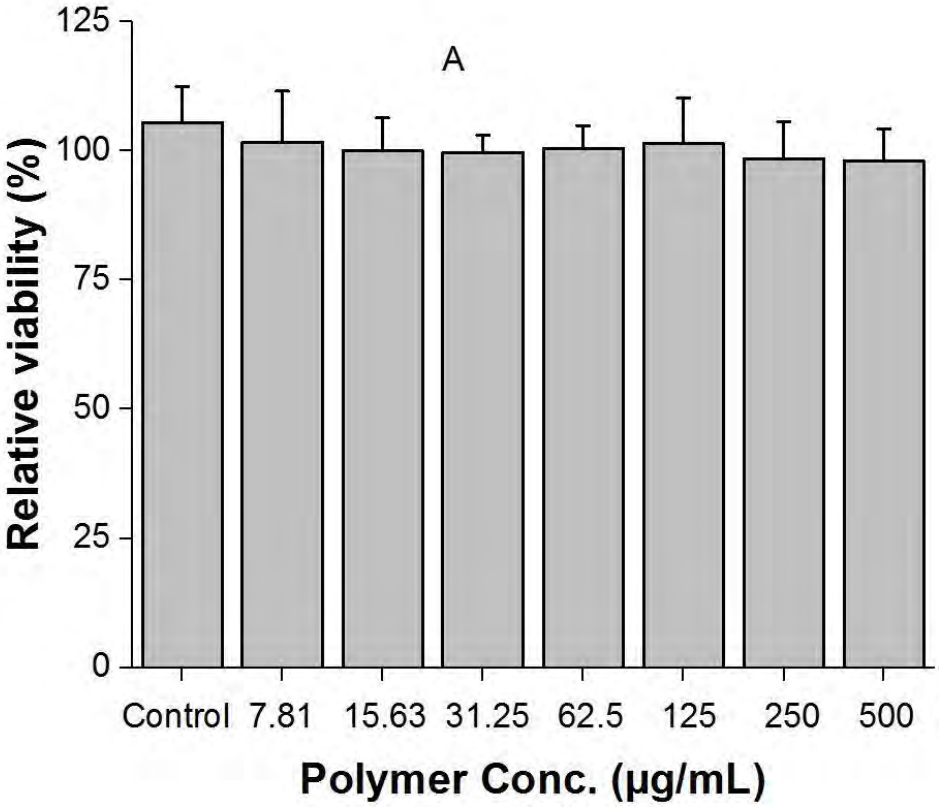


Figure 3. The fluorescent activated cell sorting (FACS) analysis of cellular uptake of Cy5.5 loaded Pep-PEG-PTMC nanoparticles after incubation with C4-2 prostate cancer cells for 3-h. Nanoparticles were pre-treated in the presence (Blue) or absence (Red) of 150 nM CTSK followed by incubation with cells at 37 ^{o}C. Blank cells without any treatment was used as a control (Black). C4-2 cells were cultured at 37 °C, treated with NPs for 3-h, washed three times with PBS, harvested and analyzed with FACSCanto flow cytometer (BD Biosciences).

The in vitro cytotoxicity of nanotherapeutics was evaluated by measuring cell proliferation using MTT assay in cultured C4-2 prostate cancer cells. After 48-h incubation period, blank polymer without inclusion of the model drug of docetaxel (DTX) showed no detectable cytotoxicity at concentrations up to 500 μg.mL^{-1} (Figure 4 A). In contrast, DTX-loaded nanotherapeutic exhibited considerable toxicity in the C4-2 cells with an IC_{50} at DTX concentration approximately 22.3 and 11.8 nM for Pep-PEG-PTMC and mPEG-PTMC, respectively, which was higher than that of free DTX (IC50 approximately at 7.2 nM) (Figure 4 B). Cell proliferation assay was also carried out when nanotherapeutics were firstly treated with 150 nM CTSK, followed by cell viability experiments. No difference was observed in the cases of free DTX or DTX-loaded nanotherapeutic by using mPEG-PTMC as the drug carrier. Interestingly, improved tumor inhibition was observed for peptide functionalized nanotherapeutics. For example, in the case of Pep-PEG-PTMC based DTX-loaded nanotherapeutics, tumor cells growth inhibition of C4-2 cells was determined as 65.7%, 42.3% and 32.7% at DTX concentration of 6.25, 25 and 100 nM, respectively. When the nanotherapeutics were pre-incubated with CTSK, the tumor cells growth inhibition of C4-2 cells was improved as 51.9%, 33.3% and 19.9% at DTX concentration of 6.25, 25 and 100 nM, respectively. (Figure 4 C). These results indicated that the drug sensitivity of peptide functionalized nanotherapeutics can be improved in a CTSK-rich microenvironment. As the progression and bone metastasis of prostate cancer are

positively correlated with expression of CTSK, it is expected that CTSK-responsive nanotherapeutics are promising for advanced prostate cancer therapy.



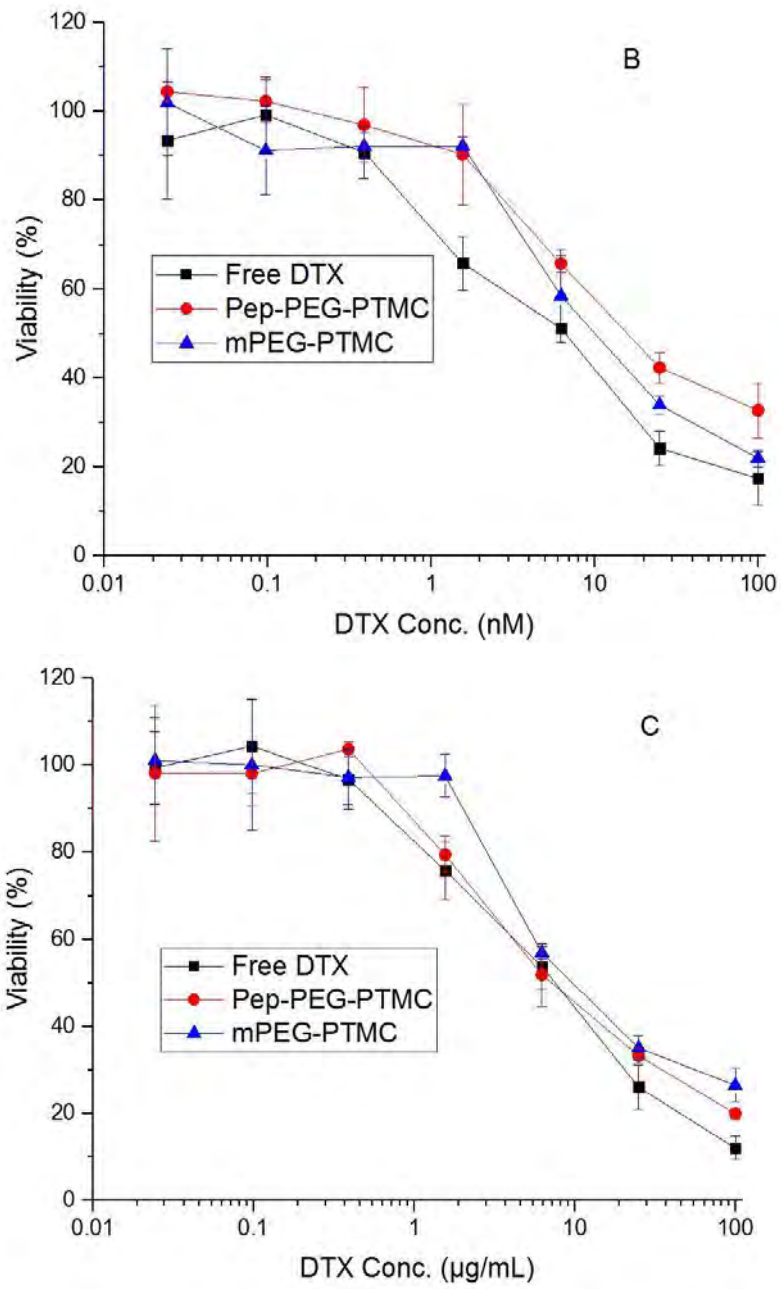
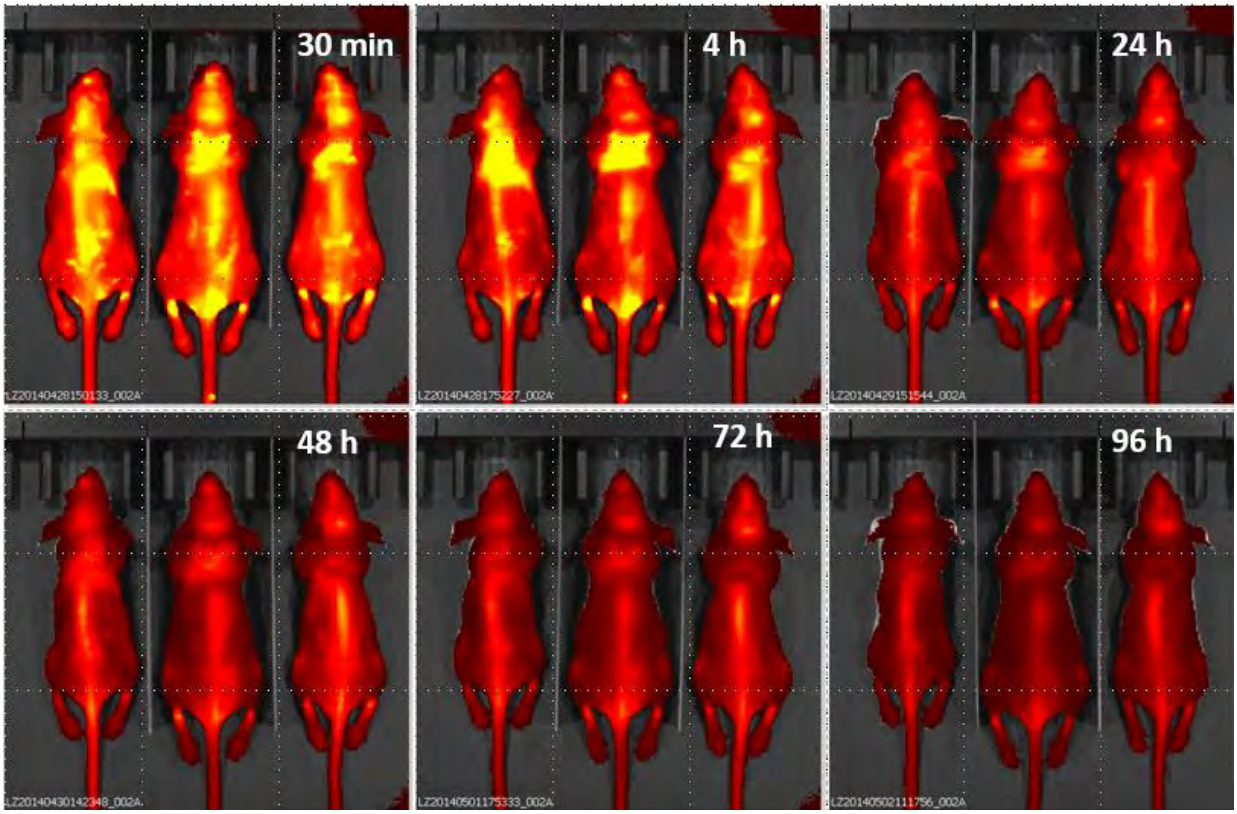


Figure 4: Cell viability of C4-2 prostate cancer cells after treatment with blank copolymer of Pep-PEG-PTMC (A). Cell viability of 4C-2 prostate cancer cells after treatment with free DTX or DTX-loaded copolymer of Pep-PEG-PTMC or mPEG-PTMC (B). Free DTX or DTX-loaded copolymer of Pep-PEG-PTMC or mPEG-PTMC was firstly treated with 150 nM CTSK followed by incubation with 4C-2 prostate cancer cells to determine cell viability by MTT assay (C).

The strong binding ability of Pep-*b*-PEG-*b*-PTMC micelles to hydroxyapatite (HA) has been confirmed that nearly 90% of the CK(DUPA)GHPGGPQAsp$_8$ modified NPs were rapidly bound to HA. In marked contrast, less than 10 % of mPEG-b-PTMC micelles were bound to HA, indicating minimal nonspecific binding to HA by the mPEG-b-PTMC micelles absent of peptide functionality. We have further studied bone uptake and retention of the nanotherapeutics in animals by using non-invasive optical imaging modality. Cy5.5 was used as a near infrared fluorescent (NIRF) optical imaging agent. Bone-targeted Cy5.5-loaded NPs were prepared using Pep-PEG-PTMC as the drug carrier. Equivalent dose of NIRF probe (2 nmol) was administered into mice via tail vein injection, and the preferential bone-tissue accumulation of Cy5.5 was clearly observed by near-infrared fluorescence imaging using bone-targeted nanocarrier in vivo and ex vivo (Figure 5). The results demonstrated that modification with osteotropic Asp8 would dramatically facilitate the specific binding towards bone tissue. This bodes well for bone-targeting capacity of nanotherapeutics *in vivo*, and more detailed examination of bone-related therapeutic evaluation will be performed in the next funding period.



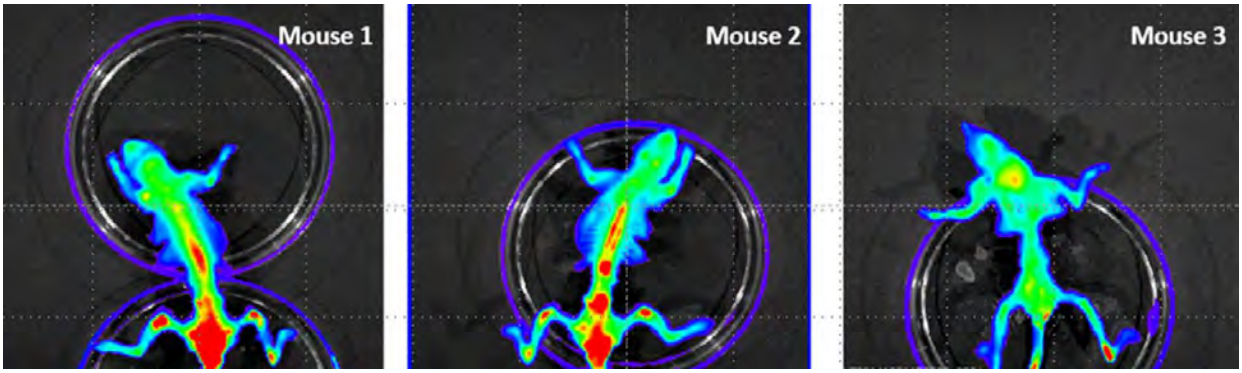


Figure 5: Athymic nude mice (Nu/Nu strain) were used for non-invasive optical imaging studies. For near-infrared fluorescence (NIRF) imaging, nude mice were intravenously injected with equivalent amount of 2 nmol Cy5.5 of bone-targeted Cy5.5-loaded NPs. At different time points post injection of imaging probe, mice were scanned using an IVIS Spectrum Pre-clinical In Vivo Imaging System (Perkim Elmer, USA) with an excitation bandpass filter at 670 nm and an emission at 720 nm. The mice were anesthetized by isofluorence during each imaging procedure. Results were analyzed using Living Image 4.4 software (Perkim Elmer, USA). After 96-h post injection of NPs, mice were sacrificed and ex vivo optical imaging was acquired to further validate that the imaging probes were indeed accumulated in hard tissues.

In summary, during the past reporting period, we have been focusing on the studies of CTSK responsiveness of nanotherapeutics *in vitro*. We have investigated and evaluated biological activities of the drug-loaded nanotherapeutics in terms of cellular uptake and tumor inhibition in cultured prostate cancer cells. We have also initiated bone uptake and retention of the proposed nanotherapeutics in mice. For the third year of this project, we will perform experiments to reveal the functionality of the ligand (i.e, DUPA) for its prostate cancer specificity. Meanwhile, we will investigate and evaluate bone-targeted nanotherapeutics in a prostate cancer animal model for therapeutic efficacy studies.

What opportunities for training and professional development has the project provided?

"Nothing to Report."

How were the results disseminated to communities of interest?

"Nothing to Report."

What do you plan to do during the next reporting period to accomplish the goals?

The progress of this project has been good during the past two years. The obtained results provided sufficient rationale and great enthusiasm to continue to test our nanotherapeutic constructs especially in animals. For the next reporting period, we will start to establish animal model of prostate cancer induced bone metastatic animal model, and perform therapeutic efficacy studies *in vivo*, such as survival study and bone-related therapeutic evaluation of bone-targeted nanotherapeutics to improve therapy for advanced prostate cancers.

4. **IMPACT:**

What was the impact on the development of the principal discipline(s) of the project?

"Nothing to Report."

What was the impact on other disciplines?

"Nothing to Report."

What was the impact on technology transfer?

"Nothing to Report."

What was the impact on society beyond science and technology?

"Nothing to Report."

5. **CHANGES/PROBLEMS:**

"Nothing to Report"

Changes in approach and reasons for change

"Nothing to Report"

Actual or anticipated problems or delays and actions or plans to resolve them

"Nothing to Report"

Changes that had a significant impact on expenditures

"Nothing to Report"

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

"Nothing to Report"

6. **PRODUCTS:**

Mnauscript:

Xuli Wang, Ye Yang, Huizhen Jia, Wanjian Jia, Scott Miller, Beth Bowman, Jun Feng and Fenghuang Zhan, Peptide decoration of nanovehicles to achieve active targeting and pathology-responsive cellular uptake for bone metastasis chemotherapy, *Biomaterials Science*, **2014**, 2, 961-971

7. **PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS**

What individuals have worked on the project?

Name:	Xuli Wang, Ph.D.
Project Role:	Principal Investigator
Nearest person month worked:	4
Contribution to Project:	Responsible for the overall management of the project. Dr. Wang has also involved in chemical synthesis for this project.
Funding Support:	This award and support from NCI
Name:	Mary Beth Bowman
Project Role:	Technician
Nearest person month worked:	3
Contribution to Project:	Ms. Bowman has performed work in the area of investigation and evaluation of nanotherapeutics.
Funding Support:	This award.
Name:	Le Zhan
Project Role:	Technician
Nearest person month worked:	6
Contribution to Project:	Ms. Zhan has performed work in the area of investigation and evaluation of nanotherapeutics.
Funding Support:	This award.

Name:	Tao Liu
Project Role:	Graduate Student
Nearest person month worked:	5
Contribution to Project:	Mr. Liu has performed work in the area of chemical synthesis and characterization of nanotherapeutics.
Funding Support:	NCI

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

"Nothing to Report."

What other organizations were involved as partners?

"Nothing to Report."

8. **SPECIAL REPORTING REQUIREMENTS**

N.A.

9. **APPENDICES:**

N.A.